

(12) UK Patent Application (19) GB (11) 2 335 599 (13) A

(43) Date of A Publication 29.09.1999

(21) Application No 9907100.3

(22) Date of Filing 26.03.1999

(30) Priority Data

(31) 9806513.9 (32) 26.03.1998 (33) GB
(31) 9905275.5 (32) 08.03.1999

(71) Applicant(s)

Phytopharm Plc
(Incorporated in the United Kingdom)
Corpus Christi House, 9 West Street,
GODMANCHESTER, Cambs, PE18 8HG,
United Kingdom

(72) Inventor(s)

Zongqin Xia
Yaer Hu
Ian Rubin
Jonathan Brostoff
Brian Anthony Whittle
Weijun Wang
Phil Gunning

(74) continued overleaf

(51) INT CL⁶

A61K 35/78 31/58 // (A61K 35/78 31:58)

(52) UK CL (Edition Q)

A5B BE BHA BJA B24X B24Y B243
U1S S2415 S2417 S2418

(56) Documents Cited

WO 99/09837 A1 WO 98/51302 A1 WO 94/18994 A1
US 5589182 A DE 4303214 A
& EPOQUE Abstract, Chemical Abstract No.
121:149054 JP090100295 A & WPI Accession No.
97-276722 [25], Chemical Abstract No. 127:39796
Chemical Abstracts No. 130:276763 & WO 99/16786
A1 Chemical Abstracts No. 122:248292, WPI Abstract
Accession No. 97-180063 [17] & CN0109292 Chemical
Abstracts No. 122:248291, WPI Abstract Accession No.
97-180062 [17] & CN0109291 Chemical Abstract No.
77:16542 and Biol. Nauki., Vol 15, No. 3, 1972, pages
85-88.

(58) Field of Search

Online: CAS ONLINE

(54) Abstract Title

Treatment of conditions characterised by a deficiency in the number and function of membrane bound receptors using saponins and sapogenins

(57) A method of treating conditions that are characterised by a deficiency in the number and function of membrane bound receptors. Such conditions include Alzheimer's disease, senile dementia (of the Alzheimer's type), Parkinson's disease, postural hypotension, chronic fatigue syndrome, Lewing-Bedy disease, Gulf War syndrome, autism, Myasthenia Gravis, Lambert Eaton disease, exposure to organo-phosphorus compounds and age-related problems of muscle and heart fibre. The mechanism of how these diseases are related to the number and function of membrane bound receptors is described in detail. Saponins and sapogenins, such as anzuogenin D, diosgenin, hecogenin, prazerigenin, ruscogenin, smilagenin, sisalogenin, sarsasapogenin, tigogenin or astragaloside, exhibit the ability to regulate receptor activity. Their ability to regulate the number and function of membrane bound receptors (muscarinic receptors) was studied in chinese hamster ovary cells and in cultured cardiac cells. Non-oestrogenic saponins and sapogenins are the preferred compounds. Saponins and sapogenins of this type were also claimed to have cognitive function enhancing properties when used to treat age-related cognitive dysfunction. These compounds may be derived from a plant of the genus Agave, Anemarrhena, Asparagus, Smilax or Yucca.

GB 2 335 599 A

(74) Agent and/or Address for Service

Haseltine Lake & Co
Imperial House, 15-19 Kingsway, LONDON, WC2B 6UD,
United Kingdom

**MEMBRANE-BOUND RECEPTORS AND THEIR FUNCTION;
COGNITIVE DISFUNCTION; TREATMENTS THEREFOR;
AND COMPOSITIONS FOR USE IN SUCH TREATMENTS**

5 The present invention relates to membrane-bound receptors and their
function; to cognitive disfunction and allied conditions; to treatments therefor;
and to compositions for use in such treatments. More particularly but not
exclusively the invention is concerned with the treatment of conditions that are
characterised by a deficiency in the number or function of membrane-bound
10 receptors. In the following, the present invention will be described principally
with reference to the treatment of Alzheimer's disease (AD) and senile dementia
of the Alzheimer's type (SDAT), where deficiencies in a number of receptor
types have been demonstrated. However, it is to be understood that the present
invention relates generally to the treatment of conditions attributable to intrinsic
15 pathological conditions and/or exposure to adverse environmental conditions
these conditions being characterised by a deficiency in the number or function
of membrane-bound receptors or a deficiency in transmission at the junctions
between neurones or at the junctions of neurones and effector cells.

20 Conditions of the type mentioned above include Parkinson's disease,
Lewi body dementia, postural hypotension, autism, chronic fatigue syndrome,
Myasthenia Gravis, Lambert Eaton disease, diseases and problems associated
with Gulf War Syndrome, occupational exposure to organophosphorus
compounds and problems associated with ageing.

25 Alzheimer's disease (AD) and senile dementia of the Alzheimer's type
(SDAT) are grave and growing problems in all societies where, because of an
increase in life expectancy and control of adventitious disease, the demographic
profile is increasingly extending towards a more aged population. Agents which
30 can treat, or help in the management of, AD/SDAT are urgently required.

Age-associated memory impairment (AAMI) is a characteristic of older patients who, while being psychologically and physically normal, complain of memory loss. It is a poorly defined syndrome, but agents which are effective in treatment of AD/SDAT may also be of value in these patients.

5 Research into AD/SDAT is being carried out by traditional and conventional medical research methods and disciplines. In conventional medicine, there are several approaches to the treatment of AD/SDAT. It is known that the biochemical processes subserving memory in the cerebral cortex are (at least in part) cholinergically-mediated. Those skilled in the art will
10 know that "cholinergically mediated" mechanisms may be directly attributable to acetylcholine acting on receptors, and these are direct effects. Other, clinically useful effects may also be caused by modulation of release of acetylcholine from pre-synaptic nerve endings or inhibition of enzymes that destroy
15 acetylcholine. These modulating factors may be exerted through neurones where the mediator is non-cholinergic; these are referred to as indirect effects. Some attempts at treatment have focussed on the role of other mediators such as 5-hydroxytryptamine, which is a mediator in other areas of brain, such as the mid-brain nuclei. However, since fibres from these areas are projected forward into the cerebral cortex where the primary transmitter is acetylcholine, attention
20 has focussed on the management of this mediator in the search for appropriate therapeutic agents.

 Cholinergic strategies for the treatment of AD/SDAT have been directed at several points along the pathway of formation, synaptic release and removal
25 of released acetylcholine.

 One approach involves treatment with high doses of lecithin and other precursors of acetylcholine. This is of limited use in producing sustained improvements in cognitive performance.

30

 Another approach involves the use of vegetable drugs such as Polygalae

root extract, which has been shown to enhance choline-acetylcholine transferase (CAT) activity and nerve growth factor (NGF) secretion in brain. Oral administration of NGF has no effect on central nervous system neurons because it is a high molecular weight protein that cannot pass through the blood-brain barrier. However, agents which can pass through the blood-brain barrier and have a stimulating effect on NGF synthesis in the central nervous system have been proposed for the improvement of memory-related behaviour.

The results of a third clinical approach, which uses cholinesterase inhibitors such as tacrine hydrochloride, have been marginally more positive than the above. Substances obtained from plants used in Chinese and Western medicine, for example huperzine, galanthamine, and physostigmine have all been shown to be of some - although limited - benefit in the treatment of AD/SDAT in clinical studies and also in laboratory models. All of these substances are inhibitors of acetylcholine esterase (AChE). In patients with AD/SDAT, there may be reduced synthesis of acetylcholine (ACh), reduced efficiency in release of ACh from presynaptic stores, and a decrease in the number or function of postsynaptic (M_1) receptors. Reductions in pre-synaptic M_2 receptors have also been shown. The beneficial effect of AChE inhibitors is attributed to enhancement of acetylcholine levels at synapses in brain by slowing down the destruction of released transmitter.

Compositions which modulate cholinergic function are known to affect memory and recall. For example, nicotine stimulates nicotinic acetylcholine receptors, and the short lived memory enhancing effects of cigarette smoking are thought to be due to the effect of nicotine. Scopolamine, an antagonist of acetylcholine, will produce amnesia and impaired cognitive function manifesting in psychomotor tests as a prolongation of simple reaction times, possibly as a result of impaired attention, and is used for this purpose as an adjunctive analgesic treatment. The amnesic effect of scopolamine can be antagonised by nicotine.

There are two families of nicotinic receptor subtypes (α and β), and each includes four subgroups which differ in ligand specificity. The role of nicotinic receptors in the CNS is not well understood at the molecular level. It is possible that agents binding to nicotinic receptors may modify the rate of turnover at muscarinic receptor sites in brain. Nicotinic receptors are ligand-gated ion channels, and their activation causes a rapid (millisecond) increase in cellular permeability to Na^+ and Ca^{++} , depolarisation and excitation.

Another class of cholinergic receptors can be stimulated by muscarine. Such muscarinic (M) receptors are G protein-coupled receptors. Responses of muscarinic receptors are slower; they may be excitatory or inhibitory. They are not necessarily linked to changes in ion permeability. Five types of muscarinic receptors have been detected by cholinergic receptor cloning, and are designated as m_1 - m_5 . Pharmacological effects are associated with four of the cloned receptors and they are designated as M_1 - M_4 based on pharmacological specificity.

Using specific receptor proteins and monoclonal antibodies, it has been possible to further localise muscarinic receptors in brain as m_1 (postsynaptic) and m_2 (presynaptic). In heart, M_2 receptors are postsynaptic. Presynaptic muscarinic receptors are thought to be inhibitory, the binding of ACh to these receptors attenuating the release of further ACh to provide a negative feedback mechanism for ACh release. Selective M_2 receptor antagonists which are preferentially distributed to the brain may therefore be useful in treating Alzheimer's disease.

It is known that, in disease states such as AD/SDAT, there is general neuronal loss and deficits in cholinergic nerve function. It has been speculated that the high affinity nicotinic binding sites in the remaining cholinergic neurons might be converted to low affinity binding sites in treating such diseases, thereby sustaining transmitter release. By lowering the affinity of the nicotinic

binding sites, a quick desensitising process is avoided.

Agonist activation at nicotinic receptors in brain has rapid onset and offset. A decreased affinity of the nicotinic receptors will reduce the
5 desensitisation process. Schwarz R.D. et al (J. Neuro Chem 42, (1984), 1495-8) have shown that nicotine binding sites are presynaptically located on cholinergic (and also 5-hydroxytryptaminergic and catecholaminergic) axon terminals. A change in high affinity binding sites on AD/SDAT may also induce a change in the modulatory effect the nicotinic binding sites may have on other transmitter
10 systems.

Presynaptic cholinergic mechanisms are also under inhibitory control by GABAergic neurons and this inhibition is thought to be intensified in AD/SDAT. Removal or reduction of this inhibition intensifies presynaptic
15 cortical cholinergic activity and enhances cognitive processing.

The interactions of interneuronal fibres innervated by nicotine (reducing binding affinity), and dis-inhibition of GABAergic fibres both have a presynaptic locus.
20

This is a simplistic model of central transmission, but provides a framework for understanding the attempts which have been made to increase the effective concentration of acetylcholine in central synapses. This further illustrates the concept of direct and indirect action. There are disadvantages
25 attaching to the three conventional therapeutic approaches to AD/SDAT treatment mentioned above: ACh precursor supplementation, agonist replacement and acetylcholine esterase inhibition. These treatments may result in a short-term increase in the availability of ACh which may activate feedback mechanisms resulting in the desensitisation of postsynaptic receptors. On
30 theoretical grounds, long term benefits would not be predicted and when treatment is interrupted, any benefits in management of AD/SDAT and AAMI

disappear and the condition may even be aggravated.

5 It has been shown that a compound with M_1 agonist and M_2/M_3 antagonist activity improved cognitive performance in SDAT patients (Sramak et al, Life Sciences vol. 2, No. 3, 195-202, 1997). However, this compound causes unacceptable cholinergic side effects, such as fatigue, diarrhoea and nausea.

10 A more radical approach to AD/SDAT and AAMI aims to increase the number of postsynaptic (M_1) receptors, in brain. It is known from Chinese Patent No. CN1096031A, that sarsasapogenin (SaG) can up-regulate M_1 cholinergic receptors and also down-regulate (i.e. move towards normal levels of) β -adrenergic receptors, the number of which may be pathologically-raised in AD/SDAT.

15 The inventors have found a number of saponins and sapogenins which exhibit the ability to regulate receptors. Thus, according to one aspect of the invention, there is provided the use of one or more of smilagenin, anzuogenin D, or an astragaloside in the manufacture of a medicament for the treatment of a
20 condition characterised by a deficiency in postsynaptic membrane-bound receptor number or function.

Those skilled in the art will be aware of the relationship between saponins and their sapogenins, and that the desired effects of sapogenins can be
25 exhibited in patients by administration of the corresponding saponins, or a mixture thereof. Hydrolysis of at least a proportion of saponin occurs in the gastrointestinal tract. The skilled man will also be aware of the epimerisation of certain sapogenins under conditions of acid hydrolysis.

30 Not all saponins and/or their aglycones are useful treatments for AD/SDAT and some, such as the saponins and sapogenins from digitalis, have

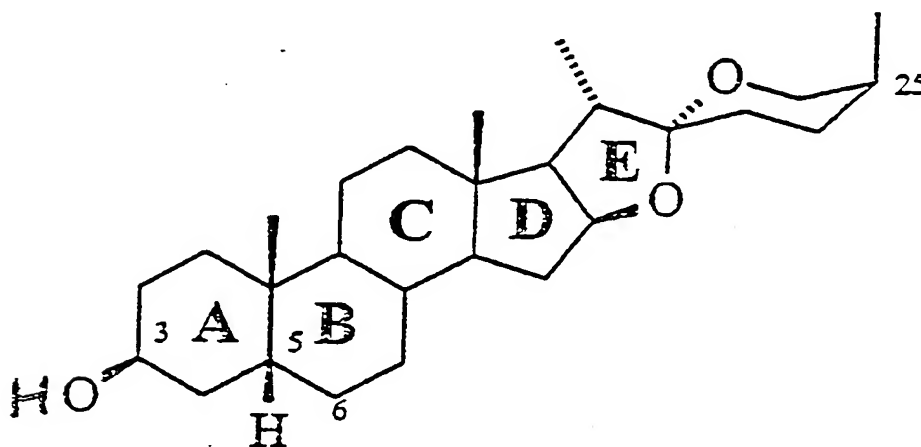
potent inotropic actions on the myocardium. This group of saponins does not appear to have effects on the central nervous system (CNS) which would predicate therapeutic use in AD/SDAT; their potency and toxicity at high doses also rule this out.

5

Some of the principal sapogenins are of the following general formula:

10

15



With reference to this general formula, the structure of certain sapogenins is as indicated in the Table below:

20

Compound	A/B ring Cis/Trans/ unsaturation	C25 methyl stereochemistry (R or S)	Hydroxyl group(s) on Spirostane ring
Sarajasapogenin	Cis	S	3 β -OH
Smilagenin	Cis	R	3 β -OH
Anzurogenin-D	Trans	R	3 β -OH, 5 α -OH, 6 β -OH
Sisalgenin	Trans	S	3 β -OH (C=O at C12)

25

Tigogenin	Trans	R	3 β -OH
Diosgenin	$\Delta 5$	R	3 β -OH
Ruscogenin	$\Delta 5$	R	1 β -OH, 3 β -OH

5

The variation in pharmacological properties and pharmacodynamic actions of various types of sapogenin underlines the need for selection of those agents which are most useful for the treatment of AD/SDAT. The discovery of novel facts about the action of SaG has made it possible to determine which substances are most useful for the treatment of AD/SDAT and the like.

10

The saponins and sapogenins of principal interest in certain aspects of the present invention occur naturally in a range of plant species, notably from the genera Smilax, Asparagus, Anemarrhena, Yucca and Agave. The species presently of greatest interest include Smilax regelii Kilip & Morton - commonly known as Honduran sarsaparilla; Smilax aristolochiaefolia Miller - commonly known as Mexican sarsaparilla; Smilax ornata Hooker - commonly known as Jamaican sarsaparilla; Smilax aspera - commonly known as Spanish sarsaparilla; Smilax glabra Roxburgh; Smilax febrifuga - Kunth - commonly known as Ecuadorian or Peruvian sarsaparilla; Anemarrhena asphodeloides Bunge; Yucca schidigera Roezl ex Orgies; and Yucca brevifolia Engelm. Saponins and sapogenins which may be of interest also occur naturally in other genera, for example Dioscorea, Trillium, Solanum, Strophanthus, Digitalis and Trigonella. As indicated above, some saponins and sapogenins from these sources possess undesirable properties and are thus not recommended for use in the invention.

15

20

25

30

According to a further aspect of the present invention, there is provided a pharmaceutical composition having cognitive function enhancing properties which comprises an effective amount of a saponin or sapogenin. The saponin or

sapogenin is preferably a steroidal saponin or sapogenin. Such a composition preferably comprises an effective amount of a non-oestrogenic saponin or sapogenin.

5 In another aspect, the invention provides a pharmaceutical composition having cognitive function enhancing properties which comprises an effective amount of a saponin or sapogenin (preferably a non-oestrogenic saponin or sapogenin) derived from a plant of the genus Smilax, Asparagus, Anemarrhena, Yucca or Agave.

10 The invention further provides the use of an extract of a plant of the genus Smilax, Asparagus, Anemarrhena, Yucca or Agave in the preparation of a medicament having cognitive function enhancing properties.

15 It will be appreciated that the invention embraces within its scope the use of the compositions defined above. Thus, according to a fifth aspect, the present invention provides a method of enhancing cognitive function which comprises administering to a human or animal an effective dosage of a composition of the invention.

20 The invention also provides a method of enhancing cognitive function in a human or non-human animal, which comprises administering an effective dose of a saponin or sapogenin, preferably a non-oestrogenic saponin or sapogenin.

25 As used herein, the term "cognitive function" refers to functions such as thinking, reasoning, remembering, imagining and learning.

30 Thus, according to a seventh aspect of the invention, there is provided the use of one or more of smilagenin, prazerigenin, an astragaloside, tigogenin, ruscogenin, hecogenin and diosgenin in the manufacture of a medicament for the

treatment of a condition characterised by a deficiency in postsynaptic membrane-bound receptor number or function.

5 The inventors have also found that when sarsasapogenin is combined with certain other sapogenins, an unexpected synergistic effect is obtained.

10 Thus, according to an eighth aspect of the invention, there is provided a composition for the treatment of a condition characterised by a deficiency in postsynaptic membrane-bound receptor number or function, the composition comprising at least two of sarsasapogenin, smilagenin, prazigerenin, an astragaloside, tigogenin, ruscogenin, hecogenin and diosgenin.

15 The substances used in the seventh and eighth aspects of the invention do not have high overt oestrogenic and/or androgenic and/or anabolic activity in patients. Nevertheless, in some embodiments, there is a low level of oestrogenic and/or androgenic supplementation.

20 According to a ninth aspect of the present invention, there is provided a method for the treatment of a condition which is characterised by a deficiency in membrane-bound receptor number or function in a tissue, organ, cell type or organelle, the method comprising:

25 modulating, directly or indirectly, the action of a cytosolic, nuclear or membrane-bound protein or receptor which, when it is activated by an agonist binding thereto, or when its activity is promoted by deactivation of an antagonist thereto, upregulates and/or normalises the number and/or turnover of membrane-bound receptors in that tissue, organ, cell type or organelle.

30 Surprisingly, the inventors have found that radiolabelled SaG is concentrated in the nuclei of brain cells isolated from rats, and that levels of M receptor mRNA are raised in rats treated with SaG. Whilst the inventors do not

wish to be bound by any theory, it is believed that SaG exerts the effects described in Chinese Patent No. CN1096031A by modulating DNA expression.

5 One possible explanation in accordance with this invention is that SaG is an intracellular agonist of a steroid receptor, possibly the oestrogen receptor, or a transcription factor or promoter. There are chemical similarities in the structure of steroids and SaG, and it is therefore possible that the transport mechanism of SaG from cytoplasm to the nucleus is the same as that for steroids. Thus, after diffusing across the cell membrane, SaG binds to a steroid
10 receptor present in the cytoplasm and promotes a conformational transformation of the receptor so that a high-affinity nuclear complex is delivered to a response site on the nuclear DNA protein complex. There, it enhances transcription of mRNA which migrates from the nucleus to the ribosomes to result in increased production of muscarinic receptors.

15 A second possibility is that SaG is an agonist of an unknown receptor, which acts to cause an increase in mRNA expression by binding to the DNA protein complex in the nucleus and acting as a promoter.

20 In either case, the binding of the SaG-receptor complex to DNA may cause an increase in the expression of mRNA which codes for cholinergic receptors, dopaminergic receptors, or adrenergic receptors or other membrane-bound receptors.

25 Alternatively, the binding of the SaG receptor complex to the DNA may cause an increase in the production of linked proteins such as G protein; or impede their degradation; or later the linkage between such proteins and associated receptors, thereby causing secondary changes in receptor number.

30 The effects of SaG may be mediated through increases in the levels of one or more neurotrophic factors, for example nerve growth factor (NGF).

It is also recognised that, in addition to the neuronal and cholinergically mediated synaptic mechanisms, it is possible that substances such as nitric oxide (NO) and non-cholinergic agonists can have a modulating effect on cholinergic transmission.

5

Whatever the precise nature of the cell component to which SaG binds in order to exert its effect, this provides a new pathway on which potential treatments for AD/SDAT, AAMI and the like can be targeted.

10

It has been shown that SaG increases the levels of membrane-bound receptor mRNA, specifically m_1 receptor mRNA. It is therefore possible that the cytosolic or nuclear receptor or promoter, when activated, increases the production of mRNA molecules in the tissue, organ, cell type or organelle which code for membrane-bound receptors, or that it decreases the breakdown of mRNA molecules in the tissue, organ, cell type or organelle which code for membrane-bound receptors.

15

20

The cytosolic or nuclear receptor, when activated, may also increase the transcription of mRNA molecules in the tissue, organ, cell type or organelle which code for membrane-bound receptors.

25

As mentioned above, nicotinic receptors may modulate the number and/or turnover of membrane-bound receptors. Accordingly, in one embodiment, the action of the cytosolic or nuclear receptor is modulated by administering a substance which is at least a partial agonist of nicotinic receptors.

30

It is presently preferred that the action of the cytosolic or nuclear receptor is modulated by administering a substance which is at least a partial agonist thereof.

The agonist may be a saponin or a sapogenin, preferably one or more of sarsasapogenin, smilagenin, prazerigenin, an astragaloside, tigogenin, ruscogenin, hecogenin and diosgenin. These compounds do not have a high overt oestrogenic and/or androgenic and/or anabolic activity in patients. A low level of oestrogenic and/or androgenic supplementation may be beneficial in the method of the ninth aspect of the present invention.

The receptor may be located in the cytosol of the cells of the tissue, organ, cell type or organelle and, when activated by binding an agonist, migrates to the nucleus of the cells. It is also possible that the receptor is located in the nucleus of the cells of the tissue, organ, cell type or organelle, the agonist diffusing into the nucleus or being transported there by another mechanism.

In the method in accordance with the first aspect of the present invention, it is not essential for an administered substance to act directly on the cytosolic or nuclear receptor itself. Instead, action can be taken either upstream or downstream of the cytosolic or nuclear receptor's or promoter's involvement in the pathway. Thus, the action of the cytosolic or nuclear receptor may be modulated by administering a substance which increases expression of the mRNA molecules in the tissue, organ, cell type or organelle which code for membrane-bound receptors.

The role of oestrogen and other related compounds as possible treatments for SDAT has received considerable interest. In the studies conducted to look at the effects of a cholinesterase inhibitor, tacrine, on cognitive function in patients with SDAT a secondary analysis suggested that all of the improvement was seen in female patients who were also receiving hormone (oestrogen) replacement therapy (ERT). Epidemiological data also suggest that ERT may protect against the development of SDAT. There is extensive work in the rat that suggests that ovariectomy results in reduced cognitive function and this effect can be reversed at least in part by the administration of oestrogen. The effects of oestrogen in

this model may be to increase high affinity choline uptake in certain areas in the brain, particularly the hippocampus, thereby improving cholinergic transmission. In the same model, administration of oestrogen has been shown to increase the levels of mRNA for brain derived neurotropic factor (BDNF) using suitable in-situ hybridisation techniques (Singh 1995).

Possible mechanisms behind the effects of oestrogen have been investigated in in-vitro experiments. These studies have been undertaken using a neuroblastoma cell line and the response of the cells to serum deprivation or the effects of beta amyloid (BA) fractions. This latter stimulus is thought to be of particular relevance because of the prominence of amyloid plaques in the late stages of SDAT. Both serum deprivation and BA induce cell death. 17- β oestradiol has been shown to protect against cell death induced by serum deprivation and BA. The protective effect was not abolished when the 17- β oestradiol was tested in the presence of the oestrogen antagonist, tamoxifen. The non-oestrogenic enantiomer, 17- α oestradiol, was as effective in inhibiting cell death. Subsequent work has suggested that the protective effects of these compounds depends on the presence of a fully de-saturated phenolic A ring and an unblocked hydroxyl group at the three position (Simpkins 1997; Green 1997). In neuroblastoma cell cultures, oestrogenic compounds were shown to increase the release of nerve growth factor. The relevance of these findings to the effects of oestrogen in SDAT remains unclear.

Patent applications have been published which claim the usefulness of a number of steroid sapogenins having spirostane, furo-spirostane, spirosolane or solanidine structures in the treatment of diseases including SDAT. Two patent publications are of particular relevance here: Chinese patent publication No CN1096031A claims the use of the spirostane sapogenin, sarsasapogenin, in the treatment of SDAT. The disclosure in this document, however, is brief. The other document of relevance is patent publication DE 4303214A1 which claims the use of a very wide range of saponins and sapogenins in the treatment of a

whole range of diseases that the inventors consider to be of viral origin. This disclosure is however of dubious value in that it is well recognised that there is no infective element to a very large number of the conditions that are characterised by deficient synaptic transmission and thus the basic premise of the alleged invention is flawed. In addition they present no data of any kind that allows one skilled in the art to be able select a preferred compound from the large number that are claimed.

In identifying compounds that would have use in the treatment of SDAT and other diseases characterised by reductions in receptor numbers or synaptic transmission, the inventors have given consideration to the need to identify compounds that would have the desired effect but would be devoid of any oestrogenic effects, as these would be unacceptable, particularly in male patients. A number of the compounds claimed to have activity in patent application DE 4303214A1 have marked oestrogenic activity and are therefore unacceptable. This data is summarised below in Table 1.

Table 1 Oestrogenic effects of steroid sapogenin compounds and selected triterpenoid

Compound	Oestrogenic Activity
Diosgenin	Positive
Anzurogenin D	Negative
Ruscogenin	Positive
Sarsasapogenin	Negative
Tigogenin	Negative
Astragaloside	Negative

Smilagenin	Negative
------------	----------

5 In addition these compounds were tested at other steroid receptors as it was considered that compounds that would be of clinical use should have no effects at the other steroid receptors. None of the compounds was found to have any activity at any of the following receptors:

10 Progesterone
Glucocorticoid
Testosterone

Thus the compounds that were shown not to have activity at the oestrogen receptor were also inactive at the other important steroid receptors.

15 The selected compounds have also been tested for their activity in a number of in-vitro assays. The assays/experiments that were considered of key importance in determining possible activity in the elevation of membrane bound receptor numbers were as follows:

- 20
1. Chinese hamster ovary (CHO) cells transfected with the a DNA fragment coding for a muscarinic receptor. The cell line used for the majority of the experiments was a cell line expressing the m2 receptor.
 - 25 2. The effects of muscarinic receptor expression in cultured cell lines of neuronal origin were investigated.
 3. Cultured cardiac muscle cells obtained from neonatal Sprague Dawley rats. The cardiac muscle cells express muscarinic receptors, typically m2.

30 The level of these receptors falls on prolonged culture and the effects of

compounds of interest in preventing the fall in receptor numbers was investigated.

The methods and the results of these experiments are now described in turn.

5

1 CHO cell line experiments

The effects of various compounds on the expression of m2 receptors on CHO cells transfected with DNA for the m2 receptor were investigated. Receptor numbers were assayed using tritiated QNB binding and subtracting non-specific binding. Compounds were dissolved in DMSO and DMSO was used as a control. Compounds were tested at a range of final concentrations. Compounds were also tested in the presence and absence of tamoxifen to try to distinguish an oestrogen receptor mediated mechanism. The results are summarised in the table 2 below.

10

15

Table 2 Effects of compounds on the expression of m_2 receptors on CHO cells

	Compound	Molar concentration of compound	Effect on receptor expression - given as % increase compared to control (negative values in brackets)
5	Sarsasapogenin	10^{-5}	34
		10^{-6}	(14)
	Anzurogenin D	10^{-5}	22
		10^{-6}	(26)
10	Sisalgenin	10^{-5}	NS
		10^{-6}	NS
	Smilagenin	10^{-5}	57
		10^{-6}	18
	Diosgenin	10^{-5}	NS
		10^{-6}	NS
15	Ruscogenin	10^{-5}	(22)
		10^{-6}	NS
	Tigogenin	10^{-5}	NS
		10^{-6}	NS

20 NS = No significant effect

Thus the experiments indicate that several of the compounds were able to increase the number of muscarinic receptors expressed on the surface of CHO cells cultured in-vitro. The effect was not antagonised by tamoxifen, indicating that the mechanism involved did not involve the oestrogen receptor. Unlike in the work published by Simpkin et al it was found that there was no need for an intact phenol A-ring. Equally a number of compounds that are steroid sapogenins were devoid of activity. Furthermore, additional experiments indicated that β -oestradiol had a similar effect in increasing receptor expression when administered at a concentration of 10^{-5} M.

2 Effects of compounds on cell survival

Other in vitro assays have been used to distinguish between active and non-active compounds. In particular various neuroblastoma cell lines including SKN-SN and SH-SY5Y cells as well as phaeochromocytoma cell lines have been cultured in vitro in the presence of β -amyloid fragments or serum depletion. A number of techniques to demonstrate the effectiveness of the compounds in protecting the cultured cells were investigated. These techniques included Trypan blue exclusion, chemiluminescence and release of lactate dehydrogenase. Of most interest was the observation that incubation of cells, in particular PC12 cells, with β -amyloid reduced the number of muscarinic receptors measured using radio-labelled ligand binding techniques. This reduction in receptor numbers was found to be ameliorated by the active compounds.

3 Effects of compounds on cultured cardiac muscle cells.

Cardiac muscle cells were isolated from the ventricular muscle of neonatal Sprague Dawley rats using standard techniques. Cells were cultured in vitro and muscarinic receptor numbers expressed on cell surfaces membrane fragments after homogenisation of cells harvested at various time points were estimated

using specific binding of tritiated QNB. Preliminary experiments demonstrated that the number of receptors expressed tended to decline after 10 days of culture. The experiments were therefore designed to investigate the effects of the various compounds in inhibiting this decline in receptor numbers.

5

The results of these experiments are summarised in Table 4:

Table 4 Effects of various compounds on muscarinic receptor expression on cultured cardiac muscle cells

10

Compound	Concentration of compound causing a significant increase in number of receptors expressed on neonatal cardiac muscle after 10 days in vitro culture
Diosgenin	NS
Anzurogenin D	10^{-6}M
Ruscogenin	NS
Sarsasapogenin	10^{-5}M
Tigogenin	NS
Astragaloside	10^{-5}M
Smilagenin	10^{-6}M

15

20

NS = No significant effect

Surprisingly the inventors have found that sapogenins are preferentially concentrated in the nuclei of cells cultured in vitro. This is surprising because,

as discussed above, sarasapogenin (SaG) and some other compounds which have been shown to increase the number of muscarinic receptors do not bind to known steroidal receptors. In addition, it is surprising that SaG is preferentially taken up into the nucleus because the effects of these compounds can be seen in
5 in-vitro assay systems that express the muscarinic receptor but where the DNA for the receptor has been transfected into the cytoplasm and hence is not under the normal nuclear control mechanism.

SaG and the other compounds that have been tested and shown to up-
10 regulate the levels of receptors, have all been shown not to bind directly to any of the major known classes of membrane bound receptor. Thus it can be postulated that the observed effects are probably not due to for instance an effect at the nicotinic receptor and a consequential increase in the number of muscarinic receptors. This explanation appears to be even less plausible
15 (although it cannot be excluded) if one considers that certain of the compounds have also been shown by the inventors to increase the number of beta adrenergic receptors expressed on peripheral blood lymphocytes. Thus the mechanism would appear to be one which has a more general effect on the regulation of membrane bound receptors.

20 It is speculated here that the effect of the active compounds claimed in this patent may operate through an effect on G protein and that the effects on receptor numbers are secondary to an effect on G-protein. When a membrane bound G-protein linked receptor is stimulated two basic sets of events are
25 initiated: the effector response; and the internalisation of the receptor. The subsequent processing of the receptor to the state where it is again in a form on the cell surface or other membrane surface where it can interact with another receptor ligand appears to be subject to a number of factors. A number of these factors or mechanisms appear to be G-protein linked. There is evidence that
30 activation of m_3 receptors may have an effect on G-protein expression or levels. It is speculated that the actions of the compounds described in this patent may

due to an interaction in the processes of receptor regeneration, G-protein linkage or G-protein homeostasis.

5 An alternative hypothesis is that the compounds are increasing the synthesis or release or a decreased rate of degradation of neurotropic factors such as brain derived growth factor and/or nerve growth factor. These effects on growth factors might be due to an effect of the compound on a cytosolic or nuclear receptor or the binding of a compound to a promoter region with a consequent effect directly on the rate of production of mRNA for the growth
10 factor or as a consequence of increasing the production of another material factor such as G-protein or finally the effects may be secondary to an effect on receptor or G-protein procession.

15 The increased expression and/or abnormal processing of the amyloid precursor protein (APP) is associated with the formation of amyloid plaques and cerebrovascular amyloid deposits which are the major morphological hallmarks of Alzheimer's disease. Of particular interest are the processes regulating the proteolytic cleavage of APP into amyloidogenic and nonamyloidogenic fragments. The cleavage of APP by the enzyme α -secretase within the β -
20 amyloid sequence of the protein results in the formation of a non amyloidogenic C-Terminal fragment, and the soluble APPs α fragment; this latter fragment has been shown to have neurotropic and neuroprotective activity as well as to enhance memory in mice when injected intra-cerebro-ventrically (ICV). In contrast, processing of APP by β -secretase exposes the N-terminus of β -
25 amyloid which is released by γ -secretase cleavage at the variable C-terminus. The resulting β -amyloid peptides, which contain 39-43 amino acids, have been shown to be neurotoxic and to accumulate in plaques which interfere with inter-neurone connections.

30 A number of studies have shown that stimulation of the protein-kinase (PKC) linked muscarinic M_1 and M_3 receptors results in an increase in α -

secretase activity. As a consequence processing of APP to APPs α with its neuroprotective effects is increased. In parallel, processing of APP by β - and γ -secretase is decreased and there is a consequential reduction of β -amyloid. Other transmitters such as nerve growth factor (NGF) and brain derived
5 neurotropic factor (BDNF) as well as bradykinin and vasopressin may have similar effects in increasing the proportion of APP processed to APPs α . There may be a number of factors involved in the effects of NGF which may include binding of the factor to the tyrosine kinase receptor (TrkA) and the stimulation of phospholipase C γ with subsequent phosphorylation and activation of protein
10 kinase C (PKC) and increase in relative activity of α -secretase.

Any treatment which increases activity of protein-kinase C selectively in brain might therefore be expected to be of use in the management of Alzheimer's disease. Until recently agonists selective at the M $_1$ receptor have not
15 been available. Non-selective agonists would be expected to stimulate pre-synaptic M $_2$ receptors which cause negative feedback and hence would further severely impair muscarinic transmission. Selective agonists at the M $_1$ receptor are now becoming available (talsaclidine) and such agents are under investigation for the treatment of AD. There is however, a substantial risk that,
20 as with the chronic administration of any receptor agonist, the clinical benefits seen will be severely limited in terms of the size of benefit by reducing receptor numbers or reducing sensitivity and in terms of side effects due to lack of receptor specificity. Thus compounds as described in this invention, which selectively increase muscarinic M $_1$ receptor numbers, with little or no effect on
25 muscarinic M $_2$ receptor numbers in the brain would be expected to be devoid of the problems seen with a muscarinic agonist and hence have particular utility. Indeed the benefits may be seen in three parts as follows.

- 30 1. A selective increase in M $_1$ receptor numbers leading to increased synaptic transmission. Chronic administration of a selective agonist will, at best, have no adverse effect on transmission;

2. Secondary to the increased receptor numbers, an increase stimulation of PKC with a consequential increase in α -secretase activity, leading to:

2.1 A reduced production of β -amyloid and a consequent reduction of plaque formation and neuronal loss;

2.2 An increase in APPs α and a consequent improvement in cerebral function as witnessed by an improvement in short and long term memory.

Finally the effects of the GABA system in modulating transmission has been discussed above. It is well know that there is a steroid binding site on the GABA receptor that is distinct from the benzodiazepine, chloride and GABA binding sites. A number of therapeutic compounds are know to bind to this site and have been used to enhance or reduce the level of consciousness. It is speculated that the chronic administration of a partial agonist at this site might lead to an enhancement of transmission.

The invention will be described further in the following example.

Example - Investigation of mRNA levels using in situ hybridisation

20 months old pure-line male SD rats were divided randomly into 2 groups. One group received an average of 3 mg of sarsasapogenin per rat per day mixed into the daily feed. The control group received normal food and water. Four months later, their brains were used in hybridisation technique experiments, with 4 to 6 months old rats used as a young control group. Other feeding arrangements for each group were completely identical.

A cDNA chain which respectively corresponds to the mRNA of both m_1 and m_2 was synthesised. m_1 corresponds to the 3-18 amino acid sequence of

receptor protein, i.e. TGG TGC CAA GAC AGT GAT GTT GGG ACT GAC
AGC AGG GGG CAC TGA GGT, and M₂ to the 1-16 amino acid sequence,
i.e. ATG AAT AAC TCA ACA AAC TCC TCG AAC AAT GGC TTG GCT
ATT ACC AGT. The cDNA was labelled using a 3'-terminal-label reagent kit
5 with α -³⁵S-dATP (8.9 TBq/mmol) as the label material. After the reaction had
finished, it was purified with a nucleotide column. The specific activity of the
batch was estimated (16.67-33.34) x 10⁸ MBq/ μ g. α -³⁵S-dATP,
3'-terminal-label reagent kit and nucleotide column were obtained from Du
Pont Co., USA.

10

One rat was obtained from each group each time and parallel
experiments were performed. The rats were decapitated and their brains
removed intact. 15 μ m thick coronal slices were prepared in a constantly
freezing cryo-microtome (AS-600 cryo-microtome, Anglia Scientific Co, UK).
15 Slices were taken from different areas (identical places for each rat) and were
mounted on slides smeared with polylysine, dried in a cool current of air, fixed
in a solution of 4% paraformaldehyde (containing 1 x phosphate buffer saline
(PBS), pH 7.0) for 5 minutes before being washed twice in PBS. They were
then placed in 0.25% acetic anhydride solution (containing 0.1 M
20 triethanolamine hydrochloride, Ph 8.0, and 0.9% sodium chloride) for 10
minutes, dehydrated in 70%, 80%, 95%, and 100% ethyl alcohol for 1 minute,
degreased in chloroform for 5 minutes, and finally treated in 100% and 95%
ethyl alcohol for 1 minute successively.

25

Slices used as negative controls were taken and dehydrated in ethyl
alcohol etc. as detailed above, but treated in advance in 100 mg/ml RNase and 2
x SSC solution (salt/sodium citrate solution containing 300 mmol/L sodium
chloride and 45 mmol/L sodium citrate) for 2 hours at 37°C.

30

For hybridisation, the fluid matrix for hybridisation was compounded
with freshly containing 50% deionised formamide, 4 x SSC, 10% dextran

5 sulphate, 250 µg/µl yeast tRNA, 5 x Denhard solution, 500 µg/ml denaturation protamine DNA, 10 mmol/L dithiothreitol. Oligonucleotide probe [(16.67-33.34) x 10 MBq/50 µl] labelled with ³⁵S was added finally and mixed evenly. 50 µl of the matrix was dripped onto each slice and a silicate cover glass was placed lightly over, avoiding airlocks. The slices were then shelved in a hybridisation box with 2 x SSC on the bottom to preserve moisture, and incubated at 37°C for 18 to 24 hours.

10 After hybridisation, the slides were soaked in 1 x SSC solution and shaken slightly to rinse the cover glass. They were washed in 1 x SSC solution briefly, then vibrated gently in 2 x SSC containing 50% formamide at 37°C for 20 minutes with the solution changed four times, and then transferred into 1 x SSC solution for vibration at laboratory temperature for 30 minutes (repeated twice). Finally, the slides were washed with double distilled water, dehydrated
15 with 70%, then 95% ethyl alcohol and dried in the air.

20 Autoradiographs were prepared in a dark room, the specimen and the hyperfilm beta max being pressed together using the contact method and placed in a cassette with a desiccant, exposed at 4°C for 2 to 3 weeks. They were developed (D196) and fixed (F5). Finally, the autoradiographs were analysed using a computerised image analyser (VIDAS imaging analyser, Kontron, Germany).

25 The m₂ probe failed to show any localised area of activity. The m₁ probe showed activity in dentate nucleus, cerebral cortex and striatum. Comparison of these three areas for the different animal groups are shown in Table 5:

Table 5

Comparison		
Area	Aged vs young	SaG vs Aged
Cortex	-5.14±2.68 (23)	5.77±3.82 (20)
Hippocampus	-3.18±2.87 (12)	0.96±4.26 (10)
Striatum	-12.2±3.6*	15.71±3.27* (10)

Positive means increased compared to the comparator.

* p<0.01. Numbers in brackets = numbers of slices.

There was a significant reduction in mRNA expression for m_1 receptors in the striatum of aged rats compared to young controls. Administration of SaG resulted in a significant increase in m_1 receptor mRNA in the same brain area when treated animals were compared to aged, untreated controls.

CLAIMS:

1. The use of one or more of smilagenin, prazerigenin, an astragaloside,
tigogenin, ruscogenin, hecogenin and diosgenin in the manufacture of a
5 medicament for the treatment of a condition characterised by a deficiency in
postsynaptic membrane-bound receptor number or function.

2. The use of smilagenin, anzuogenin D or an astragaloside in the
manufacture of a medicament for the treatment of a condition characterised by a
10 deficiency in postsynaptic membrane-bound receptor number or function.

3. A composition for the treatment of a condition characterised by a
deficiency in postsynaptic membrane-bound receptor number or function, the
composition comprising at least two of sarsasapogenin, smilagenin, prazerigenin,
15 an astragaloside, tigogenin, ruscogenin, hecogenin and diosgenin.

4. A pharmaceutical composition having cognitive function enhancing
properties which comprises a pharmacologically effective amount of a saponin
or sapogenin.
20

5. A pharmaceutical composition as claimed in claim 4, wherein said
saponin or sapogenin is a steroidal saponin or sapogenin.

6. A pharmaceutical composition having cognitive function enhancing
25 properties which comprises a pharmacologically effective amount of a non-
oestrogenic saponin or sapogenin.

7. A pharmaceutical composition having cognitive function enhancing
properties which comprises a pharmacologically effective amount of a saponin
30 or sapogenin derived from a plant of the genus Smilax, Asparagus,
Anemarrhena, Yucca or Agave.

8. A pharmaceutical composition having cognitive function enhancing properties which comprises an effective amount of a non-oestrogenic saponin or sapogenin derived from a plant of the genus Smilax, Asparagus, Anemarrhena, Yucca or Agave.

9. The use of an extract of a plant of the genus Smilax, Asparagus, Anemarrhena, Yucca or Agave in the preparation of a medicament having cognitive function enhancing properties.

10. A method of enhancing cognitive function which comprises administering to a human or animal an effective dosage of a composition as claimed in any one of claims 1 to 8.

11. A method of enhancing cognitive function in a human or non-human animal, which comprises administering an effective dose of a non-oestrogenic saponin or sapogenin.

12. A method of enhancing cognitive function in a patient suffering from age-related cognitive disfunction, which comprises administering to the patient a pharmacologically effective dose of a non-oestrogenic saponin or sapogenin.

13. A method for the treatment of a condition which is characterised by a deficiency in membrane-bound receptor number or function in a tissue, organ, cell type or organelle, the method comprising:

modulating, directly or indirectly, the action of a cytosolic, nuclear or membrane-bound protein or receptor which, when it is activated by an agonist binding thereto, or when its activity is promoted by deactivation of an antagonist thereto, upregulates and/or normalises the number and/or turnover of membrane-bound receptors in that tissue, organ, cell type or organelle.

14. A method as claimed in claim 13, wherein said protein or receptor, when activated, increases the amount of mRNA molecules in the tissue, organ, cell type or organelle which code for membrane-bound receptors.

5 15. A method as claimed in claim 13 or claim 14, wherein said protein or receptor, when activated, increases the production of mRNA molecules in the tissue, organ, cell type or organelle which code for membrane-bound receptors.

10 16. A method as claimed in claim 13, 14 or 15, wherein said protein or receptor, when activated, increases the transcription or expression of mRNA molecules in the tissue, organ, cell type or organelle which code for membrane-bound receptors.

15 17. A method as claimed in claim 13, 14, 15 or 16, wherein said protein or receptor, when activated, decreases the breakdown of mRNA molecules in the tissue, organ, cell type or organelle which code for membrane-bound receptors.

20 18. A method as claimed in any one of claims 13 to 17, wherein said protein or receptor, when activated, modulates the expression of DNA in the tissue, organ, cell type or organelle which code for membrane-bound receptors.

25 19. A method as claimed in claim 14, 15, 16, 17 or 18, wherein the action of said protein or receptor is modulated by administering a substance which increases expression of the mRNA molecules in the tissue, organ, cell type or organelle which code for membrane-bound receptors.

30 20. A method as claimed in any one of claims 13 to 19, wherein said protein or receptor, when activated, directly or indirectly upregulates and/or normalises the number and/or turnover of muscarinic receptors in that tissue, organ, cell type or organelle.

21. A method as claimed in any one of claims 13 to 20, wherein said protein or receptor, when activated, directly or indirectly normalises and/or downregulates the number and/or turnover of adrenergic receptors in that tissue, organ, cell type or organelle.

5

22. A method as claimed in any one of claims 13 to 21, wherein the action of the receptor is modulated by administering a substance which is at least a partial agonist of nicotinic receptors.

10

23. A method as claimed in any one of claims 13 to 22, wherein the action of said protein or receptor is modulated by administering a substance which is at least a partial agonist of the receptor.

15

24. A method as claimed in claim 23, wherein the agonist is a pharmaceutically acceptable saponin or sapogenin.

20

25. A method as claimed in claim 24, wherein the agonist is one or more of sarsasapogenin, smilagenin, prazerigenin, an astragaloside, tigogenin, hecogenin, ruscogenin and diosgenin.

25

26. A method as claimed in any one of claims 13 to 25, wherein the receptor is located in the cytosol of the cells of the tissue, organ, cell type or organelle and, when activated, migrates to the nucleus of the cells.

27. A method as claimed in any one of claims 13 to 25, wherein the receptor is located in the nucleus of the cells of the tissue, organ, cell type or organelle.

30

28. A method as claimed in any one of claims 13 to 27, wherein the receptor is a steroid receptor.

29. A method as claimed in any one of claims 13 to 28, wherein the receptor is an oestrogen receptor.

5 30. A method as claimed in any one of claims 13 to 29, which is for the treatment of Alzheimer's disease or a senile dementia of the Alzheimer's type.

31. A method as claimed in claim 13 and substantially as hereinbefore described.

10 32. A composition for use in the method of claim 13, substantially as hereinbefore described.



Application No: GB 9907100.3
Claims searched: 1-32

Examiner: Dr Lawrence Cullen
Date of search: 2 July 1999

Patents Act 1977 Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:
UK CI (Ed.Q):
Int CI (Ed.6):
Other: Online: CAS ONLINE

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X	US 5589182 A (TASHIRO et al.) see col 2, line 32, to col 3, line 63; see Tables 1 & 2; Examples 2-5	4-13, 24, 30
X, P	WO 99/09837 (CV TECHNOLOGIES) see Table, page 10, and formula, page 12; see lines 3-12, page 15; line 3, page 29 to line 31, page 32/2	4-6, 10-13, 24, 30
X	WO 98/51302 A1 (UNIV. of WASHINGTON) see page 1, line 6 to page 2, line 22; page 4, lines 31-32; ; page 9, lines 30-31; Claim 6	4-6, 10-13, 24, 30
X	WO 94/18994 A1 (PHARMAKON) see page 2, lines 1-9; page 3, line 20; page 6, lines 26-31	4-6, 10-13, 24, 30
X	DE 4303214 A (MARKS) & EPOQUE Abstract, Chemical Abstract No. 121:149054, see Abstracts, Structures Ib of Fig. 1, Fig. 2 & Table, page 5.	3-6, 10-13, 24, 25
X	JP 090100295 A (TORAY) & WPI Accession No. 97-276722 [25], Chemical Abstract No. 127:39796, see Formula I and abstracts	4-6, 10-13, 24, 30

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application



Application No: GB 9907100.3
Claims searched: 1-32

Examiner: Dr Lawrence Cullen
Date of search: 2 July 1999

Category	Identity of document and relevant passage	Relevant to claims
X	Chemical Abstracts No. 130:276763 & WO 99/16786 A1 (INST. RADIATION MEDICINE), see Abstract & Formulae I & II.	1-13, 24, 25, 30
X	Chemical Abstracts No. 122:248292, WPI Accession No. 97-180063 [17] & CN 109292 A (SHENYANG), see Abstract and Formula	4-6, 10-13, 24, 30
X	Chemical Abstracts No. 122:248291, WPI Accession No. 97-180062 [17] & CN 109291 A (SHENYANG), see Abstract and Formula	4-6, 10-13, 24, 30
X	Chemical Abstract No. 77:16542 and Biol. Nauki. Vol 15, No. 3, 1972, D. Guseinov et al., pages 85-88; see Abstract	1, 3-6, 10-13, 24, 25

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application